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(54) Title: PRIMERS FOR THE AMPLIFICATION OF GENES CODING FOR THE ENTEROTOXIN AND THE LECITHINASE OF CLOSTRIDIUM PERFRINGENS AND THEIR APPLICATION TO THE DETECTION AND NUMERATION OF THESE BACTERIAE

(57) Abstract

The presence of lecithinase or enterotoxin genes or the presence of Clostridium perfringens bacteriae in a sample is determined by polymerase chain reaction using specific primers. The bacteriae are detected in particular in food samples or fecal samples.

Primers for the amplification of genes coding for the enterotoxin and the licithinase of Clostridium perfringens and their application to the detection and numeration of these bacteriae

The present invention relates to primers for the amplification of genes coding for the enterotoxin and the lecithinase , also called phospholipase C, of Clostridium perfringens.

Another object of the invention is the application of these primers for the detection and the numeration of C. perfringens.

Clostridium perfringens type A is widely distributed (soil, sewage, intestinal tracts of humans and animals), and is a common causative agent of bacterial food poisoning outbreaks worldwide. The symptoms, predominantly diarrhea and abdominal pain, appear 6 to 24 hours after ingestion of contaminated food. Vomiting and fever are unusual. Death occurs occasionally among debilitated persons, particularly the elderly.

C. perfringens enterotoxin CPE which is produced during the sporulation phase has been shown to produce the symptoms associated with C. perfringens food poisoning. The illness is caused by the ingestion of food that contains larger number of vegetative enterotoxigenic C. perfringens (more than 10^5 organisms per g) . These bacteriae multiply and sporulate, releasing CPE into the intestine.

A C. perfringens count of more than 10^6/g in fecal samples of ill persons is indicative of C. perfringens food poisoning . In addition, CPE detection directly in fecal samples is a valuable method confirming the diagnosis.

1

The epidemiological investigations involve C. perfringens numeration in suspected foods. The characterization of the enterotoxigenic C. perfringens strains is not used routinely, since C. perfringens

5   sporulates poorly in usual culture media.

Recently, CPE and phospholipase C gene sequences have been determined (VAN DAMME et al. 1989, Ant. Van Leeuwen 56, 181-190; TSO J. and SIEBEL, 1989. Infect. Immun. 57: 468-476, TITBALL et al. 1989 Infect. Immun.

10   57:367-376). The phospholipase C gene is located on a variable region of the chromosomal DNA in all C. perfringens toxinotypes whereas the distribution of CPE gene is restricted. Only 6% of the C. perfringens isolates from various origins showed the presence of CPE gene by

15   DNA-DNA hybridization. This ratio is higher (59%) among C. perfringens strains isolated from confirmed outbreaks of food poisoning. In the standard methods, the enterotoxin detection by biological or immunological tests requires previously the sporulation of C. perfringens. Several

20   specific medium and protocols for C. perfringens sporulation have been described, but they are time consuming and many C. perfringens strains do not sporulate or very poorly (DUCAN and STRONG , 1968. Appl. Microbiol. 16: 82; PHILIPS , 1986 , Lett. Appl. Microbiol 3: 77-79),

25   which impairs the CPE detection.

A method for the detection of C. perfringens by polymerase chain reaction (PCR) has yet been disclosed during the third Congress of the French Society of Microbiology (April 21-24, 1992) through a poster of FACH

30   et al.

The method was consisting in an amplification of the parts of the genes encoding the α-toxin , also called

2

lecithinase, and the enterotoxin of C. perfringens by using oligonucleotidic primers which were choosen in these genes. However, the sequence of the primers used for carrying out this method was not disclosed and the

5   sensitivity mentioned by the authors was low , i.e. from 500 to 5000 bacteriae per gram of feces.

Moreover, the results were obtained on feces artificially contaminated and not on feces from contaminated patients or on contaminated food.

10   The inventors have thus sought to elaborate a sensitive and reliable method allowing the detection of low quantities of bacteriae in samples of different origins, such as in feces or food, in a raw form.

They have surprisingly shown that it is necessary

15   to choose the primers in some well determined regions of the genes, as well as in the gene of the enterotoxin that in the one of the lecithinase.

Besides , they have carried out a process for the treatment of foods samples, allowing a specific, sensitive

20   and reliable determination of the presence of the C. perfringens contained in these foods.

The present invention firstly relates to primers for the amplification of the gene encoding the lecithinase of C. perfringens, also called alphatoxin or phospholipase

25   C, corresponding to a part of the sequence of the gene comprised between the nucleotides 1350 and 1850, and preferentially between nucleotides 1350 and 1470 or 1650 and 1850, and which can amplify at least a part of the said gene, by cooperating with other primers having

30   similar features and having a reversed polarity.

The said primers comprise prefentially from 10 to 40 and more preferentially from 10 to 30 nucleotides and

3

their sequences can be one of the following ones :
SEQ ID1 (PL3)   AAG TTA CCT TTG CTG CAT AAT CCC
SEQ ID2 (PL7)   ATA GAT ACT CCA TAT CAT CCT GCT
SEQ ID3 (Plc)   TCA AAA GAA TAT GCA AGA GGT
5   SEQ ID4 (PL1)   TTCTAT CTT GGA GAGG CTATGCAC
SEQ ID5 (PL4)   GCTACTAGTTCTTTTACATTCTTTCC.

The invention relates besides to primers for the
amplification of the gene encoding enterotoxin of C.
perfringens, corresponding to a part of the sequence of
10   the gene comprised between the nucleotides 450 and 950 and
preferentially between the nucleotides 450 and 550, or the
nucleotides 750 and 950 of said gene, and which can
amplify at least a part of the said gene, by cooperating
with other primers having similar features and having a
15   reversed polarity.

The said primers comprised prefentially from 10 to
40 and more preferentially from 10 to 30 nucleotides and
their sequences can be one of the following ones:
SEQ ID6 (P145)   GAA AGA TCT GTA TCT ACA ACT GCT GGT CC
20   SEQ ID7 (P146) GCT GGC TAA GAT TCT ATA TTT TTG TCC AGT
SEQ ID8(Ent A) GAA CGC CAA TCA TAT AAA TTT CCA GCT GGG

The present invention relates moreover to a process
for the determination of the presence of the gene encoding
the lecithinase, or of the gene encoding the enterotoxin,
25   in a sample, comprising the following steps:
        - the DNA from the sample is isolated ,
        - parts of the genes encoding the lecithinase, or
the enterotoxin, are amplified by polymerization chain
reaction (PCR) with the help of specific primers, such as
30   defined hereabove respectively for the lecithinase and for
the enterotoxin,
        - the amplification products are determined with

4

the help of known methods.

-       The present invention is further directed to a new C. Perfringens beta toxin gene, called β_2 gene, as hereinbelow described.

5   Another object of the present invention is a process for the determination of the presence, and the numeration of C. perfringens in a sample, wherein:

        - the DNA from the said sample is isolated ,

        - parts of the genes encoding the lecithinase and
10   the enterotoxin are amplified by polymerase chain reaction with the help of specific primers such as defined hereabove for respectively the lecithinase and the enterotoxin ,

        - the amplification products are determined with
15   the help of known methods.

        The amplification products obtained by the process described hereabove can in particular be determined by electrophoresis on an agarose gel, followed by a transfert of the eluted DNA on "Nylon" membranes and by an
20   hybridation with a labelled probe specific of the amplified part of the gene.

        Other methods known by the man skilled in the art can also be used , if such methods are sufficiently specific and sensitive.

25   Such methods are in particular described in "The molecular cloning : A Laboratory Manual; SANDERS et al., Cold Spring Harbor Editor", in which the man skilled in the art can refer for carrying out the processes according to the present invention.

30   The choice of the hereabove described primers did not obviously emerge from the genes sequences such as published. Indeed, the man skilled in the art well knows

5

that the choice of primers for the amplification of a given DNA part is difficult and that one can be faced with numerous difficulties.

5   For example, primers can lack of specificity or be weakly thermodynamicly stable.

The man skilled in the art can also be faced with other technical difficulties from different types. Thus, the choice that has to be made by the man skilled in the art in the sequence of the gene is difficult because of 10   the important number of thinkable combinations between the two primers corresponding respectively to sequences of the two strands, from the DNA molecule which has to be amplified.

The use of softwares help the man skilled in the 15   art in his choice but does not constitue a method leading automatically and obviously to primers permitting the sought amplification.

The primers such as defined hereabove are able to amplify the said genes if they cooperate with another 20   primer having similar features but of reversed polarity. Indeed, the other primer must be situated on the strand of reversed polarity , called non-sense strand, in such a way that the polymerization of the two strands, initiated from the two primers produce two types of single strand DNA 25   fragments which hybridate and form a double strand DNA molecule, called amplification product, which will be determined by known methods. The primer cooperating in the amplification of the fragment of the said gene have some similar features, i.e. that it is situated in the region 30   of the said gene but on the strand of reversed polarity.

Preferentially, the combinations of the primers can be the following : PL1 and PL4, PL3 and PL7, P145 and

6

P146, P145 and Ent A.

- The processes hereabove described allow the detection of C. perfringens in a lot of samples, such as feces or food products, such as convenients foods, without 5 necessiting a pretreatment of the sample.

In a general way, heating of the samples at temperature lysing the bacteriae and separating by centrifugation the DNA from bacterial fragments is sufficient.

10 In the case of food samples, in particular meats, it can be necessary to perform a pretreatment of the sample in order to eliminate substances which can interfer with the polymerase chain reaction.

Thus, the DNA can be isolated from a food sample by 15 a process comprising the following steps:

- the sample is incubated in a medium allowing the growth of C. perfringens,

- the bacteriae are separated from the food particles by centrifugation,

20 - the bacteriae are put in contact with a resin which lyses them ,

- the DNA is separated from bacterial fragments.

The present invention also relates to a new C. perfringens beta toxine gene which has been identified, 25 isolated and characterized.

Said gene, called β_2 gene, can be isolated from the C. Perfringens strains of C-type. Two corresponding plasmids were filed on December 12, 1994 at the CNCM Collection of Institut Pasteur and were registered 30 respectively under the reference I-1499 for TGI/pMRP 109 and under the reference I-1500 for TGI/pMRP 126.

7

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      1 · AAA GAA ATG GAT GCG TAT AGG AAA GTG ATG GAG AAT TAT CTT AAT GCT        40
      1   K   E   M   D   A   Y   R   K   V   M   E   N   Y   L   N   A          16

     49 ·TTA AAA AAC TAC GAT ATT AAT ACA GTT GTA AAC ATT TCA GAA GAT GAA        96
     17   L   K   N   Y   D   I   N   T   V   V   N   I   S   E   D   E          32

     97 AGA GTA AAT AAT GTT GAA CAG TAT AGA GAA ATG TTA GAA GAT TTT AAA        144
  5  33   R   V   N   N   V   E   Q   Y   R   E   M   L   E   D   F   K          48

    145 TAT GAT CCT AAC CAA CAA CTG AAA TCT TTT GAA ATA CTT AAT TCA CAA        192
     49   Y   D   P   N   Q   Q   L   K   S   F   E   I   L   N   S   Q          64

    193 AAG AGC GAT AAT AAA GAA ATA TTT AAT GTA AAA ACT GAA TTT TTA AAT        240
     65   K   S   D   N   K   E   I   F   N   V   K   T   E   F   L   N          80

    241 GGT GCA ATT TAT GAT ATG GAA TTT ACT GTA TCA TCT AAA GAT GGA AAA        288
 10  81   G   A   I   Y   D   M   E   F   T   V   S   S   K   D   G   K          96

    289 TTA ATA GTA TCT GAT ATG GAA AGA ACA AAA GTT GAG AAT GAA GGA AAA        336
     97   L   I   V   S   D   M   E   R   T   K   V   E   N   E   G   K         112

    337 TAT ATT TTA ACA CCA TCA TTT AGA ACT CAA GTT TGT ACA TGG GAT GAT        384
    113   Y   I   L   T   P   S   F   R   T   Q   V   C   T   W   D   D         128

    385 GAA CTA GCA CAA GCA ATT GGG GGA GTT TAT CCA CAA ACA TAT TCT GAT        432
 15 129   E   L   A   Q   A   I   G   G   V   Y   P   Q   T   Y   S   D         144

    433 AGA TTT ACA TAT TAT GCA GAT AAT ATA TTA TTA AAC TTC AGA CAA TAT        480
    145   R   F   T   Y   Y   A   D   N   I   L   L   N   F   R   Q   Y         160

    481 GCA ACT TCA GGT TCA AGA GAT TTA AAA GTA GAA TAT AGT GTT GTA GAT        528
    161   A   T   S   G   S   R   D   L   K   V   E   Y   S   V   V   D         176

    529 CAT TGG ATG TGG AAA GAT GAT GTT AAA GCT TCT CAA ATG GTA TAT GGT        576
 20 177   H   W   M   W   K   D   D   V   K   A   S   Q   M   V   Y   G         192

    577 CAA AAT CCT GAT TCT GCT AGA CAA ATA AGA TTA TAT ATA GAA AAA GGA        624
    193   Q   N   P   D   S   A   R   Q   I   R   L   Y   I   E   K   G         208

    625 CAA TCT TTC TAT AAA TAT AGA ATA AGA ATT AAA AAC TTT ACA CCT GCA        672
    209   Q   S   F   Y   K   Y   R   I   R   I   K   N   F   T   P   A         224

    673 TCA AT
    225   S
 25
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pMRP109 is a recombinant plasmid containing the beginning of the beta toxin 2 gene.

pMRP109 was produced by insertion into the pUC19
30   vector restricted by SmaI of a DNA fragment amplified by PCR from the total DNA of strain CWC 245 of C. perfringens type C and primers deduced from the N-terminal protein

8

sequence of beta 2 (P279) and an internal protein sequence
(P280):

P279 5'-AAA GAA ATI GAT GCI TAT AGI AAA GTI ATG G-5'

P280 5'-AT IGA IGC IGG IGT AAA ATT-3'.

5   The complete DNA inserted into pMRP109 was
sequenced. This DNA corresponds to the beginning of the
beta toxin 2 gene and codes for the first 225 amino acids
of the functional protein.

pMRP126

10   GAT   528
                                                                      D   176

     529 CAT TGG ATG TGG AAA GAT GAT GTT AAA GCT TCT CAA ATG GTA TAT GGT   576
     177 H   W   M   W   K   D   D   V   K   A   S   Q   M   V   Y   G   192

     577 CAA AAT CCT GAT TCT GCT AGA CAA ATA AGA TTA TAT ATA GAA AAA GGA   624
     193 Q   N   P   D   S   A   R   Q   I   R   L   Y   I   E   K   G   208

15   625 CAA TCT TTC TAT AAA TAT AGA ATA AGA ATT AAA AAC TTT ACA CCT GCA   672
     209 Q   S   F   Y   K   Y   R   I   R   I   K   N   F   T   P   A   224

     673 TCA ATT AGA GTA TTT GGT GAA GGG TAT TGT GCA TAG AAA AAA ATA TGA   720
     225 S   I   R   V   F   G   E   G   Y   C   A   *   C-terminal   236

     721 AGT GAC TTA GTC ACT TCA TAT TTT TTT TAC TAT TAA TTT TAT TAT ATA   768

     769 AAA ACC TAA CAT ACA TGA AAG TAT TCT TAA TAC AGT TAT ATC AAA ATT   816

20   817 AAA GTA GGG GAA ATA AAA TAA AAG GCT AAA AAC TAT ATT AAA AAC TAT   864

     865 AAA AAT TAT TAA ATT AGG   882

     pMRP126 is a recombinant plasmid containing the end
25   of the beta 2 gene.
     pMRP126 was produced by cloning a DNA fragment of
about 400 base pairs cleaved by Sau3A from C. perfringens
type C strain CWC245 in the pUC19 vector cleaved by BamHI.
     This part of the beta 2 gene codes for amino acids
30   176 to 236. Nucleotides 526 to 677 are common with the
last 151 nucleotides of pMRP109.
     The present invention is illustrated without being

9

limited by the following examples in which figure 1 is an
ethidium bromide stained agarose gel of amplification
products obtained with enterotoxigenic C. perfringens type
A strain 8-6 (1), type D strain IP76 (2), type E strain
5   NCIB10748 (3), and non enterotoxigenic C. perfringens type
A strain ATCC13124 (4), type B strain CN3922 (5) and type
C strain CWC236 (6) (Panel A), and Southern hybridization
with Plc (Panel B), and EntA (Panel C) probes.
MATERIALS AND METHODS
10   Bacterial strains

All bacterial strains used in this study are listed
in table 1. The others C. perfringens strains were
isolated from food poisoning outbreaks and feces of
children suffering from diarrhoea (see table 1).
15   Clostridium were grown, under anaerobic conditions,
in TYG medium: trypticase (30 g/l), yeast extract (20
g/l), glucose (5 g/l) and cysteine HCl (0.5 g/l), pH 7,2.
Strains were confirmed as C. perfringens by lactose
fermentation, nitrate reduction, gelatinase production and
20   absence of motility.
Standard bacteriological methods

For bacteriological food and feces analysis, 10g of
the samplee were weighted aseptically into sterile
stomacher bags and homogenized for 2 minutes with 90 ml of
25   peptoned water. One ml of decimal dilutions of the
suspension was mixed with 9 ml SPS agar: Tryptone (15
g/l), yeast extract (10 g/l), feric citrate (0.5 g/l),
sodium sulfite (0.5 g/l), sodium thioglycolate (0.1 g/l),
tween 80 (0.005 g/l), polymyxine B sulfate (0.001 g/l),
30   disodic sulfadiazine (0.12 g/l) and agar (14 g/l pH 7).
After a 18 h anaerobic incubation at 46°C the number of
sulfito-reductor Clostridium was determined. Colonies

10

surrounded with the black characteristic precipitate were
identified by biochemical test (lactose fermentation,
nitrate reduction, gelatinase production and motility).

Anti C. perfringens enterotoxin immunoglobulins

5   C. perfringens enterotoxin was purified from C.
perfringens strain 8-6 and rabbit anti C. perfringens
enterotoxin antibodies were prepared as previously
described (POPOFF, 1984, FEMS MICROBIOL Let.21: 1-5). Anti
C. perfringens enterotoxin immunoglobulins were purified
10   by immunoaffinity. C. perfringens enterotoxin (3- 5 mg)
was coupled to 1 g of cyanogen bromide - activated
Sepharose 4B (Pharmacia, Paris, France) according to the
instructions of the manufacturer. Five ml of rabbit anti
C. perfringens enterotoxin serum were passed over the
15   immuno affinity column. The column was washed with
phosphate-buffered saline (PBS) until no protein was
detected, and eluted with 1 M acetic acid. Fractions of
200 µl were collected into tubes containing 100 µl TRIS 3
M pH 8. The fractions containing anti C. perfringens
20   enterotoxin immunoglobulins were dialyzed against PBS.

Preparation of latex beads

          Coating of latex beads (Bacto Latex 0.81, Difco,
USA) was performed as described (SHAHRABADI et al. 1984,
Clin. Microbiol. 20: 339-341). Latex suspension (2 - 5 ml)
25   was diluted in 15 ml of glycine buffer (0.1 M glycine,
0.15 M NaCl, pH 8.2) containing 200 µg of specific anti C.
perfringens enterotoxin immunoglobulins, and homogenized
for 1 minute at room temperature. Then, 1 volume of PBS -
0.1 % bovine serum albumine (BSA) was added. The mixture
30   was shaken by vortex mixing, and stored at 4°C. The
negative latex control was prepared as the same, but using
non immune IgG (Sigma, Paris, France).

<center>11</center>

<u>Slide latex agglutination test (SLAT) for C. perfringens</u>
<u>enterotoxin detection.</u>

SLAT was performed on a glass slide by mixing 25 μl
of coated latex and 25 μl of serial ten fold dilutions of
5   samples in PBS - 0,5 % BSA.

The mixture was gently rotated and the results were
recorded after 3 minutes.

The enterotoxin detection in C. perfringens strains
was carried out using SLAT and culture supernatant from
10   cultures in the Duncan and Strong sporulation medium
(DAMME-JONGSTEN et al. J. Clin. Microbiol. 28: 131-133).

Fecal specimens were diluted at 1 : 10 in PBS-0.5 %
BSA, homogenized by vortexing, and centrifuged at 15 000 x
g for 30 minutes. The clarified supernatant was tested by
15   SLAT.

<u>Primers used in duplex PCR</u>

The oligonucleotides for PCR amplification and for
hybridization used in this study, the primers PL3, PL7,
PL1, PL4 and Plc were derived from the C. perfringens
20   alpha-toxin gene (11), the primers P145 and P146 and the
probe Ent A were from the enterotoxin gene (VAN DAMME et
al. 1989, précédemment cités).

All the oligonucleotides were chemically
synthesized by using a nucleic acid synthesizer (model 380
25   B; Applied Biosystems Inc. Foster City, Calif.).

<u>Duplex PCR with broth culture</u>

One ml of overnight bacterial cultures were
transferred to a microcentrifuge tube and centrifuged at
12.000 x g for 3 minutes. The pellet was resuspended in
30   100 μl water, heated at 100°C for 10 minutes, and
centrifugated at 12000 x g for 10 minutes. Five μl of the
supernatant were submitted to PCR using a PTC-100

12

programmable Thermal Controller (M.J. Research Inc. Watertown, MA, USA). Each reaction tube contained 50 μl of a solution of 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl$_2$, 0.1 mg/ml gelatine, 200 μM of each of the deoxynucleotide (Boehringer Mannheim, Germany), 0.5μM of each primer and DNA sample. Evaporation within the tube was prevented by the addition of 100 μl mineral oil (Sigma, St-Louis, MO, USA). The reaction mixtures were treated to 94°C for 5 minutes to denature the DNA and then 2.5 units of Taq polymerase (Boehringer) were added to each tube. All PCR reaction were performed using 45 cycles of the optimized thermal profile: 30 s denaturation at 94°C , 30 s primer-annealing at 55°C and 30 s primer-extension at 72°C. After the 45th cycle, the extension reaction was continued for another 10 minutes at 72°C, to ensure that the final extension steps was complete. Negative controls containing all reagents except template DNA were performed in each amplification set. To avoid contamination, sample preparation, PCR amplification and electrophoresis were performed in three different rooms.

Duplex PCR with stool samples

Stool samples (0,1 g) were weighed aseptically into sterile tubes and homogenized with 0,9 ml of water. Samples were centrifugated at 12.000 x g for 3 minutes. The pellet was resuspended in 100 μl water, heated at 100°C for 10 minutes, centrifugated at 12000 x g for 10 minutes, and 5 μl of the supernatant were PCR amplified as described above.

Pretreatment of food samples before the amplification reaction.

a) Incubation of the sample.

The detection of viable bacteriae is of great

13

importance in the food processes.

The discrimination between dead and alived bacteriae can be performed by cultivating the sample.

5   For the detection of C. perfringens, 25g of the sample are incubated in 225 ml of buffered peptonized water for 18 hours at 37°C in anaerobiosis and without shaking.

The spores of C. perfringens are selected with the help of a thermal shock at 80°C of the food culture before
10   incubating.

b) <u>Treatment of the sample for the PCR analysis.</u>

After incubation of the food cultures, the food fragments are sedimentated and 1 ml of the enrichment medium is taken at the surface.

15   A low speed centrifugation (600 rpm) allows the elimination of the lowest food particles. The supernatent is recovered and centrifugated at a high speed (12 000 rpm) for 2 minutes . The supernatant is eliminated and the bacterial pellet is suspended in 2 ml of sterile water.
20   The tube is then centrifuged at 12 000 rpm and the supernatant is eliminated with the help of a Pasteur pipette .

200 μl of the "InstaGene" matrix (commercialized by Biorad) are added on the pellet and incubated at 56°C for
25   30 minutes. The tube is vortexed during 10 seconds and put in a water bath at 100°C during 8 minutes. The tube is then vortexed for 10 seconds and centrifuged at 12 000 rpm for 3 minutes. 3 μl of the supernatant are taken in order to perform the duplex PCR which is finally performed on a
30   volume of 50 μl.

Starting from 25 g of foods containing 10 or more than 10 C. perfringens allows the determination in the 15

14

μl of the amplified solution of the amplification product.

Gel Electrophoresis

A 10 μl aliquot of amplified DNA was examined after electrophoresis through a composite agarose gel consisting of 3% NuSieve agarose and 1 % SeaKem agarose (FMC BioProducts, Ropckland, (Me, USA) and containing 0.5 μg/ml ethidium bromide, in 1 x Tris-borate-EDTA buffer (0.089 M Tris, 0.089 M bioric acid, 0.002 M EDTA, pH 8). Electrophoresis was carried out for 1 hour a t 80 V. The gel was examined visually with an ultra-violet transilluminator.

Hybridization procedure

DNAs electrophoresed on agarose gel were transferred to "Nylon" membranes (Boehringer, Paris, France) using 0.4 N NaOH and fixed by heating the membranes for 15 minutes at 120°C. Filters were pre-hybridized in 5 x Saline Sodium Citrate buffer (SSC), 0.1 % N-laurylsarcosine, 0.02 % Sodium Dodecyl Sulfate (SDS) in 1 % blocking reagent (Boehringer) at 65°C for 1 hour. Hybridization occurred with Ent A or Plc oligonucleotide 3'-endlabelled by digoxigenin 11-DUTP with terminal deoxynucleotidyl-transferase (Boehringer). The probe was added to 50 ng/ml in the pre-hybridization solution and filters were incubated at 40°C for 1 hour. After 2 x SSC, 0.1% SDS washing, membranes were washed to a final stringency of 50°C in 0.1 x SSC, 0.1 % SDS and detection was performed with alkaline phosphatase-labelled anti-digoxigenin antibody (Boehringer), nitroblue tetrayolium (NBT) and bromo-4-chloro-3-indolyl phosphate (BCIP) according to the instruction of the manufacturer (Boehringer).

EXAMPLE 1:

<u>C. perfringens duplex PCR specificity and characterization of the enterotoxigenic strains.</u>

The specificity of the duplex PCR with the two primers sets derived from the phospholipase C and CPE genes was assessed by using different Clostridium species and various bacterial species frequently encountered in foods (table 1). The 283 bp amplification product was observed in ethidium bromide stained agarose gel with all C. perfringens strains tested (table 1). This amplification product had the expected size of the phospholipase C gene DNA fragment generated by PL3 and PL7 primers set. This result was confirmed by DNA-DNA hybridation with the internal primer Plc (figure 1).

The other bacterial strains tested showed no amplification products with the two primers sets PL3-PL7 and P145-P146 (table 1).

Seven C. perfringens strains out of 24 showed an additional 426 bp amplification product which corresponded to the expected size of the CPE DNA fragment delimited with P145 and P146. This 426 bp amplification product hybridized with the Ent A primer localized between P145 and P146 on the CPE DNA sequence and not with Plc primer derived from the phospholipase C gene (figure 1).

The C. perfringens strain 8.6 was found enterotoxinogenic by the duplex PCR amplification (table 1). This data is in agreement with the fact that this strain produced CPE using Vero cell cytotoxicity , mouse lethality and immunoprecipitation.

EXAMPLE 2:

<u>Sensitivity of the duplex PCR using broth cultures.</u>

The duplex PCR was performed directly with broth cultures of the enterotoxogenic C. perfringens strain 8.6,

16

in order to determine the sensitivity of the method. The number of bacteriae in culture samples was determined microscopically using a Petrov Chamber. The PCR amplification was carried out as described in material and
5   methods. It was found that as few as 50 bacteria in the reaction volume yielded positive results after agarose gel electrophoresis and ethidium bromide staining. The sensitivity was 10 fold increased when the amplification products electrophoresed on agarose gel were transferred
10   to nylon membrane and hybridized with digoxigenin labelled internal probes Ent A and Plc. In these condituions, the detection limit was approximately 5 bacteria in the reaction volume of the duplex PCR.

EXAMPLE 3

15   Application of the duplex PCR to stool samples.

Twenty three stool samples originated from a suspected C. perfringens food borne intoxication outbreak were investigated directly by the duplex PCR and standard microbiological methods for C. perfringens detection.
20   Eighteen samples were found positive in the duplex PCR (table 2). All these samples yielded two amplifications products which has the expected size of the DNA fragments delimited by PL3-PL7 and P145 - P146, and hybridized with the internal primers Plc and Ent A respectively,
25   signifying that they contained enterotoxigenic C. perfringens. The sulfito reductor bacteria counted by the standard bacteriological methods ranged from less than 10^4 to 10^8 bacteria per gram. Five feces samples were found negative in the duplex PCR and DNA-DNA hybridization test.
30   The corresponding sulfito-reductor bacteria counting was less than 10^4 per gram for 3 samples, and 10^5 to 2.10^5 for the two other (table 2).

17

EXAMPLE 4

C. perfringens enterotoxin SLAT in stool samples.

The CPE was detected in 18 stool samples out of 23 by SLAT as described in material and methods (table 2).

5   Enterotoxigenic C. perfringens was identified by the duplex PCR in 17 of these 18 SLAT positive stool samples. Four samples were negative with both tests.

One stool sample was shown to contain enterotoxigenic C. perfringens by the duplex PCR and no

10   CPE by SLAT. The low number of enterotoxigenic C. perfringens (less than 10^4 per gram) explained the absence of CPE in this example.

CPE was detected by SLAT in one stool sample (table 2) which was negative in the duplex PCR. The level

15   of sulfito reductor bacteria in this sample was 10^5 per gram. However, twenty C. perfringens clones isolated on sheep blood agar, were identified as enterotoxigenic C. perfringens by the duplex PCR.

EXAMPLE 5:

20   Application of the duplex PCR to naturally and artificially contaminated food samples.

The food sample which was responsible of the food intoxication outbreak was found to contain enterotoxigenic C. perfringens by the duplex PCR performed directly

25   without enrichment culture. The counting of sulfitoreductor bacteria was 10^5 per g, and no CPE was detected by SLAT.

In order to investigate the sensitivity of this method, the duplex PCR was assayed directly with

30   artificially contaminated food samples. The detection limit was found to be 10^5 C. perfringens per g. The sensitivity was improved by using an overnight enrichment

18

culture as described in material and methods. Among 59 naturally contaminated food samples, 2 were found to contain 5.10^5 and 10^3 C. perfringens per g respectively by the classical method and were positive by the duplex PCR.

5   All the 57 food samples which did not contain sulfitoreductor bacteria and were artificially contaminated (10 C. perfringens per g) gave a positive result in duplex PCR after enrichment culture (table 3).

CONCLUSION

10   All the 24 C. perfringens strains tested yielded the 426 bp DNA fragment hybridizing with the probe Plc, which corresponds to the presence of the phospholipase C gene. Seven of these C. perfringens strains (2 type A reference strains, 3 type A strains isolated from food,

15   and 2 type D strains) showed an additional amplification product hybridizing with Ent A probe, which indicates the presence of the CPE gene.

The duplex PCR is specific of C. perfringens. No amplification product was observed with 27 Clostridium

20   strains different from C. perfringens and 20 other bacteria species frequently encounted in foods.

An important advantage of this method is to identify easily and quickly the enterotoxigenic C. perfringens strains.

25   The duplex PCR can be used with C. perfringens grown in regular culture medium for anaerobic bacteria without DNA purification. The sensitivity of the duplex PCR using pure culture is 50 bacteria by visualization of ethidium bromide stained amplification product in agarose

30   gel, and 5 bacteria in the reaction volume by DNA-DNA hybridization with the internal probes.

This duplex PCR DNA-DNA hybridization assay was

19

tested with biological and food samples. Enterotoxigenic
C. perfringens were detected directly in 18 among 23
stools samples from patients suffering food borne
intoxication. These results were in agreement with the
numeration of the sulfito reductor bacteria by the
standard method and the detection of the CPE by SLAT,
except for two stools samples. One stool sample containing
10^5 sulfito reductor bacteria per gram and CPE by SLAT was
found negative by the duplex PCR. But, individual C.
perfringens clones isolated from this stool sample were
identified as enterotoxigenic C. perfringens by the duplex
PCR. The presence of PCR inhibitors in this sample
accounts probably for this negative result. The other
stool sample showed a low number of sulfito-reductor
bacteria and no detectable CPE, but was positive in the
duplex PCR. A contamination of this sample could explain
the positive result in duplex PCR.

The detection limit of enterotoxigenic C.
perfringens in stools by the duplex PCR is 10^4 and 10^5 per
gram. This sensitivity is suitable for the diagnosis of C.
perfringens food borne intoxication, since it has been
reported that C. perfringens is recovered at high number
(10^6 and more per g) in stools from patients suffering C.
perfringens food borne intoxication.

Controls of food C. perfringens contamination
constitute an important step in the prevention of food
born intoxication by this microorganism. The standard
method routinely used detects sulfito-reductor bacteria
which encompasse C. perfringens and also other
Clostridium. The duplex PCR is specific of C. perfringens
and discriminates the enterotoxigenic strains which are
the causative agents of C. perfringens food borne

20

intoxication. The sensitivity of this method (10 C. perfringens per g) performed with enrichment cultures of food samples is compatible with the prescribed detection levels for food controls. The limit of the duplex PCR

5   directly with food samples without enrichment cultures is 10^5 bacteria per g , the duplex PCR can be used directly when a C. perfringens food borne intoxication is suspected.

The duplex PCR is more rapid and simple than the

10 standard procedure which needs additional bacteriological tests for the identification of the suspected clones. This method can be applied to food controls for the detection of C. perfringens and identification of the enterotoxigenic strains.

15 EXAMPLE 6:

Further experiments illustrating the application of the duplex PCR according to the invention to the detection of C. perfringens in foodstuffs, are presented hereinbelow.

20   1) ANIMAL FEED

Several samples of animal feed have been analyzed for the presence of C. perfringens. These feeds were made up of meat, fish or bone meals. The concentration of sulfito-reductor anaerobic organisms cultivated at 46°C

25 have been determined in 44 naturally contaminated foods and more precisely the presence of C. perfringens spores has been ascertained by use of LS medium (BEERENS H, Romond Ch., Lepage C., Criquelion J. (1982). A liquid medium for the enumeration Clostridium perfringens in food

30 and faeces. In "Isolation and identification methods for foods poisons organisms", pp. 137-149. Ed. Corry J.E.L., Roberts D. and Skinner F.A. Academic Press London) and by

21

the PCT duplex technique described previously. Spore
selection was carried out by thermal shock treatment at
80°C for 10 minutes. The presence of inhibitors for the
gene amplification reaction (PCR) led to use an extraction
5   and purification procedure for the nucleic acids which
involved the "Insta Gen" (Bio-Rad) matrix and which has
been described previously.

On the 44 samples analyzed (table 4) 12 were
positive according to the procedure using the LS medium
10   and the PCR duplex test. 24 samples were negative with
both methods. 36 samples out of the 44 analyzed gave the
same results for the two methods, giving a correlation
rate of over 80% between the bacteriological method and
the genetic test. For 5 samples the PCR duplex method
15   seemed to be more sensitive than the procedure using the
LS medium. For these 5 samples it was possible to isolate,
from the thioglycolate liquor incubated for 48 hours, at
least one colony on TSC medium which had the biochemical
characteristics of C. perfringens. The low concentration
20   of C. perfringens (< 10/g) in the samples was only
detected by the PCR technique, which was more sensitive
than the procedure using thee LS medium.

Three samples were found to be positive with the
procedure using LS medium but stayed negative with the PCR
25   duplex test. These samples contained respectively 7.2 and
1 ASR per gram. In order to ascertain whether the PCR
duplex test had not been compromised by the presence of
inhibitors remaining after the nucleic acid extraction
phase, a few nanograms of C. perfringens DNA (2 ng) were
30   added to the extracts obtained from the 3 samples and the
gene amplification test was again carried out under the
same conditions. The 3 tests then became positive showing

22

that no inhibitor effect had been observed initially. In addition, the 10 ASR colonies corresponding to these 3 samples were subjected to biochemical identification and a PCR duplex test. These 2 analyses still remained negative,

5   showing evidence of the absence of C. perfringens in these samples. In fact, bearing in mind the difficulties in interpretation of the method described by BEERENS and al. (the presence of gas in 1/3 of the Durham belljar and the presence of a black precipitate at the bottom of the tube

10   were sometimes evaluated differently according to the users) and the very doubtful results obtained for these 3 samples, it seems that these 3 samples correspond to false positives in the method described by BEERENS and al.

15   2) **HUMAN FOODSTUFFS**
        Within the framework of systematic monitoring of food hygiene, the number of C. perfringens which can be identified is generally low (< 10^2 per gram). Various foods (cooked dishes, beef , cooked meats, fish, powdered

20   milk) were analyzed using the PCR duplex technique and the results were compared with those obtained by the standard bacteriological method (standard method ISO 7937).
        Only 3 samples were found to be positive according to the two methods, the other 27 containing no C.

25   perfringens. The PCR test allowed the detection of C. perfringens in 24 hours, while the bacteriological method was much longer (48-72 h). The foods concerned were packs of a frozen ground beef and potato dish. They were contaminated by type A non-enterotoxinogenic C.

30   perfringens.
     EXAMPLE 7:
          STUDY OF THE C. PERFRINGENS BETA TOXIN GENE

23

In order to apply a PCR type method to the identification of type C C. perfringens strains responsible for necrotic enteritis, it was necessary to determine the sequence of the beta toxin gene.

5   The applicant carried out the production and purification of the beta toxin from the CWC 245 strain of C. perfringens, then determined peptide sequences, produced oligonucleotide markers and finally carried out the cloning and sequencing of this gene.

10   (1) - **Production and purification of the beta toxin**.

Cultures were made on TGY (Trypticase 30 g/l, yeast extract 20 g/l, glucose 25 g/l, pH 7.5) in a 2 l fermenter with continuous nitrogen flow, moderate stirring and regulation of pH at 7.5.

15   After 5 hours at 37°C, cholesterol in alcoholic solution (20 mg/l) was added to the culture in order to precipitate the theta toxin. The culture was centrifuged at 10 000 g at 4°C for 20 minutes. The supernatant was precipitated by 60% saturated ammonium sulfate. After

20   centrifuging at 20 000 for 20 minutes, the residue was dissolved in PBS (pH 6.8) and reduced with 50 mM of dithiothreitol. The sample was added in 3 fractions to a biogel P4 (Bio-rad) column equilibrated in PBS. The eluted proteins were applied to a column of thiopropyl-Sysharon

25   6B (58g of resin expanded in PBS) and a flow of 5 ml/h. The column was washed with 100 ml of PBS. Under these conditions the beta and delta toxins were not retained on the column, while the theta and alpha toxins were retained. The effluent was concentrated by ultrafiltration

30   on a PM 10 (Millipore) membrane and submitted to preparative iso-electrofocalization (16 000 V for 16 hours) with a pH gradient of 3.5 to 8. The material

focalized between pH 5.3 and 5.5 was collected and
concentrated by ultrafiltration.

The concentrated fraction was added to a column of
Biogel P100 (Biorad) equilibrated in PBS and the different
5   fractions were tested by electrophoresis in
polyacrylamide-SDS gel. In this way the beta toxin was
purified to homogeneity (MW 28 000 and pH 5.4 - 5.5).

The purified beta toxin was lethal to mice (4 µg,
IV), dermonecrotic after injection into guinea-pig skin (2
10   µg), and enteronecrotic after injection into rabbit
intestinal loop (40 µg). The enterotoxic effect was
inhibited by incubation of the beta toxin with trypsin.

(2) Protein microsequencing of the beta toxin and
oligonucleotides.

15   The purified beta toxin was electrophoresed on
polyacrylamide-SDS, then transferred onto Immobilon
membrane and subjected to microsequencing of the N-
terminal region.

The internal protein sequences were obtained by
20   tryptic digestion of the beta toxin, purification of the
different peptides by HPLC chromatography and
microsequencing of peptides 21, 23 and 13.

Oligonucleotides deduced from the protein sequences
were synthesized taking into account of the normal codon
25   of Clostridium and with inosines (I) in the most
degenerate positions (figure 2).

(3) Hybridization of the oligonucleotides.

The synthetized oligonucleotides were tested for
hybridization with total DNA of CWC 245 digested by
30   different restriction enzymes. The hybridizations were
carried out over 16 hours at 40°C with oligonucleotides
labeled with ^{22}P by polynucleotide kinase and washings

25

were carried out in 6 X SSC at 40°C.

- After autoradiography, these oligonucleotides did not clearly reveal a specific DNA fragment. However, clonings were carried out and the clones, identified with
5   the help of the oligonucleotides , did not contain the DNA fragments sought for after verification by nucleotide sequencing.

(4) <u>Amplification of a DNA fragment of the beta toxin gene by PCR</u>.

10   Since the production of recombinant clones containing the beta toxin gene was not possible by conventional cloning methods, this gene was amplified by PCR. To do this, an oligonucleotide (P 279) deduced from the N-terminal protein sequence of the beta toxin and an
15   oligonucleotide (P 280) deduced from an internal protein sequence (peptide 13) but in the reverse direction were used.

A DNA fragment of about 600 bp was thus amplified specifically from the total DNA of CWC 245.

20   The 600 bp DNA fragment was cloned in the pUC19 vector (pMRP 109 recombinant plasmid) and then sequenced. It contained an open reading framework and the terminal portions corresponded to the protein sequences obtained by protein microsequencing (N-terminal and peptide 13),
25   showing that the cloned DNA fragment corresponded well to the gene sought for.

The 3' part of this gene was cloned in pUC 19 using the total DNA of CWC 245 digested by Sau 3A and identified with the P 311 marker whose sequence is localized in the
30   3' part of insert of pMRP 109. The pMRP 126 recombinant clone was sequenced and contained the stop codon of the beta toxin gene and a consensus transcription termination

26

sequence. The sequence of the beta toxin gene from strain CWC 245 and its translation into amino acids are given in figure 3.

   (5) <u>Comparison of the beta toxin gene from strain</u>
5 <u>CWC 245 with that from strain NCTC 8533 and genetic</u>
   <u>analysis of the type C strains.</u>

   Titball et al (FEMS Microbiology Letters 97 (1992) 77-82) recently published the cloning and sequencing of the beta toxin gene from C. perfringens type B strain NCTC
10 8533.

   Comparison of the protein sequence of the beta toxin from strain NCTC 8533 (provisionally referred to as beta 1) with that of the beta toxin gene from strain CWC 245 (provisionally referred to as beta 2) showed no
15 significant homology (figure 4).

   The role of beta toxin 2 as a virulence factor has not yet been clearly established.

   The presence of the beta 1 and beta 2 genes was searched for by PCR in a series of type B and C C.
20 perfringens strains (Table 1).

   PCR conditions:
   - Buffer :
         - TRIS 10 mM pH 8.2
         - KCl 50 mM
25   - dNTP 100 mM
         - Bovine serum albumin 0.1 mg/ml
         - Beta-mercaptoethanol 10 mM
         - Primers 50 pmoles of each per reaction
         - TAQ polymerase (Amersham) 0.5 µ
30 - Primers:
         - Primers for beta 2
         - P319 5'-GGAAAGTGATGGAGAATTATCTTAATGC-3'

27

-    P320   5'-GCAGAATCAGGATTTTGACCATATACC-3'
(reverse)
                573 bp amplified fragment.
        - Primers for beta 1
5   - BetatoxL 5'-AGGAGGTTTTTTTATTTTTTTTTTTGAAG-3'
        - BetatoxR 5'-TCTAAATAGCTGTTACTTTGTG-3'
                962 bp amplified fragment.
        - Amplification cycles
                - initial denaturation, 2 min at 94°C
10   - 30 cycles comprising -20 s at 94°C
                                        - 20 s at 50°C
                                        - 20 s at 72°C
                - 5 minutes at 72°C
                PREM III thermocycler apparatus (Flobio)
15   -   Amplification   products   analysis   by
electrophoresis   on   1 %   agar   gel   containing   ethidium
bromide and detection by UV transillumination.

        These   strains   were   identified   by   the   standard
method   on   mice   using   anti   C.   perfringens   neutralizing
20   serum   (Welcome)   according   to   the   currently   recognized
nomenclature; all these strains produced the beta toxin.

        Genetic   analysis   showed   that   the   3   B   strains
possessed the beta 1 gene and that the type C strains fell
into 3 groups: those with the beta 1 gene, those with the
25   beta 2 gene, and those with both (table 5).

        The above experimental results show that the beta
toxin gene from strain CMC245 differs from the beta toxin
gene from strain NCTC 8533.

28

TABLE 1

Ability of duplex PCR to distinguish enterotoxigenic C.perfringens
and C. perfringens between other Clostridium and other enterobacteria.

Bacteria	Strain	PCR Result (1)
Clostridium perfringens type A	ATCC 13124	+
Clostridium perfringens type B	CN 39.22	+
Clostridium perfringens type C	CWC 236	+
Clostridium perfringens type D	2534	+
Clostridium perfringens type D	250	+
Clostridium perfringens type D	A0	+
Clostridium perfringens type D	48	++
Clostridium perfringens type D	76	++
Clostridium perfringens type D	64/1	++
Clostridium perfringens type E	NCIB 10748	++
Clostridium perfringens	8-6	++
Clostridium perfringens	1088.0	++
Clostridium perfringens	4012	++
Clostridium perfringens	4086	++
Clostridium perfringens	4009	+
Clostridium perfringens	4010	+
Clostridium perfringens	4011	+
Clostridium perfringens	1089.1	+
Clostridium perfringens	1089.2	+
Clostridium perfringens	1089.3	+
Clostridium perfringens	1089.4	+
Clostridium perfringens	1089.5	+
Clostridium perfringens	1122	+
Clostridium perfringens	1513	+

Bacteria	Strain	PCR Result (1)
Clostridium spiroforme	247	
Clostridium subterminale	ATCC 25774	
Clostridium septicum	ATCC 12464	
Clostridium limosum	384	
Clostridium beijerinckii	VPI 5481	
Clostridium mangenotii	ATCC 25761	
Clostridium chauvei	IP 91	
Bacillus thuringiensis	14001	
Bacillus subtilis	168	
Bacillus cereus	LCHA 1203	
Listeria monocytogenes 1/2 a	A3	
Listeria monocytogenes 1/2 b	15	
Listeria monocytogenes 4 b	03	
Listeria innocua 6 a	C3	
Listeria welshimeri 6 b	E3	
Listeria seeligeri 1/2 b	D3	

TABLE 1 (continued)

Ability of duplex PCR to distinguish enterotoxigenic C. perfringens and C. perfringens between other Clostridium and other enterobacteria

Bacteria	Strain	PCR Result (1)
Clostridium botulinum type A	ATCC 25763	-
Clostridium botulinum type B	F 11	-
Clostridium botulinum type C	468	-
Clostridium botulinum type D	1873	-
Clostridium botulinum type E	9009	-
Clostridium botulinum type F	NCIB 10658	-
Clostridium botulinum type G	NCIB 10714	-
Clostridium baratii (tox -)	ATCC 27639	-
Clostridium baratii (tox F)	ATCC 43756	-
Clostridium butyricum (tox -)	CB 25-1	-
Clostridium butyricum (tox E)	ATCC 43181	-
Clostridium tetani	E 19406	-
Clostridium sordellii	IP 82	-
Clostridium bifermentans	ATCC 638	-
Clostridium innoccuum	NCIB 10674	-
Clostridium sporogenes	ATCC 3584	-
Clostridium perapurificum	ATCC 25780	-
Clostridium difficile	ATCC 9689	-
Clostridium difficile	203-1	-
Clostridium oedematicus	ATCC 17861	-

Bacteria	Strain	PCR Result (1)
Escherichia coli 0 : 157 H7	LCHA P1	-
Escherichia coli 0 : 6 H16	LCHA 877	-
Escherichia coli 0 : 125 B15	LCHA 483	-
Salmonella typhimurium	3612	-
Salmonella enteritidis	3625	-
Salmonella agona	3626	-
Proteus sp	720S	-
Kurthia sp	C4	-
Pseudomonas aeruginosa	32	-
Staphylococcus aureus	C3-0	-
Yersinia enterocolitica	S31	-

(1) Symbols used for PCR results :
- : Negative reaction for alpha-toxin and enterotoxin genes.
+ : Positive reaction for alpha-toxin gene.
+ + : Positive reaction for alpha-toxin and enterotoxin genes.

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Table 2: Stool samples investigation

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Samples	Number of sulfito-reductor bacteria	Duplex PCR	SLAT
1	$< 10^4$	-	-
2	$< 10^4$	-	-
3	$< 10^4$	-	-
4	$< 10^4$	+	-
5	10^4	+	+
6	10^4	+	+
7	10^4	+	+
8	2.10^4	+	÷
9	5.10^4	+	+
10	5.10^4	÷	÷
11	10^5	-	÷
12	10^5	+	+
13	10^5	÷	÷
14	2.10^5	-	-
15	2.10^5	+	+
16	8.10^5	÷	+
17	10^6	÷	÷
18	2.10^6	÷	÷
19	2.10^6	÷	÷
20	10^7	÷	÷
21	10^7	+	+
22	10^7	+	+
23	10^7	÷	+

31

Table 3. C. perfringens investigation in food samples

Food sample	C. perfringens investigation in naturally contaminated food samples			C. perfringens investigation in artificially contaminated food samples	
	Total	Classical method	Duplex PCR	Total	Duplex PCR
Cooked food	31	1 (5×10^5)	1	30	30
Pork butchery	13	1 (10^3)	1	12	12
Raw meat	11	0	0	11	11
Milk product	2	0	0	2	2
Salad	2	0	0	2	2

32

TABLE 4:

Comparison between LS medium and the PCR
technique in the detection of C. perfringens

5   N = 44 animal foods
         (bone meal, meat or fish meal)

		LS MEDIUM (Beerens)	
		+	–
10 PCR	+	12	5 *
	–	3**	24

* ASR concentration < 10/g

     At least 1 colony identified as C. perfringens
after isolation on TSC medium.

15   ** No colony isolated on TSC medium was identified as C.
perfringens.

TABLE 5

PCR identification of the genes for beta toxin 1
sequenced from strain NCTC 8533 and beta toxin 2
sequenced from strain CWC 245 in different strains of type
B and C C. perfringens identified by the standard method

	Beta 1	Beta 2
C. perfringens B		
NCTC8533	+	−
NCTC6121	+	−
IP278-220	+	−
CN3922	+	−
C. perfringens C		
ATCC3628	+	−
NCTC8081	+	−
NCTC3180	+	+
NCTC3182	+	+
NR866	+	+
NR316	+	+
CWC236	+	+
CWC239	+	+
CWC240	+	+
CWC241	+	+

34

TABLE 5 (continued)
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5   PCR identification of the genes for beta toxin 1
           sequenced from strain NCTC 8533 and beta toxin 2
   sequenced from strain CWC 245 in different strains of type
   B and C C. perfringens identified by the standard method

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	Beta 1	Beta 2
CWC243	+	+
IP232-87	+	+
IP692-83	+	+
CWC235	−	+
CWC237	−	+
CWC245	−	+
CWC249	−	+
S608	−	+
IP93R	−	+

WHAT WE CLAIM IS:

1. A primer for the amplification of at least a part of a gene encoding the lecithinase from C. perfringens, characterized in that it corresponds to a part of the sequence comprised between nucleotides 1350 and 1850 and preferentially between nucleotides 1350 and 1470 or nucleotides 1650 and 1850 of the said gene, and in that it is able to amplify a part of the said gene, by cooperating with another primer having similar features and a reversed polarity.

2. A primer according to claim 1, characterized in that it comprises almost from 10 to 30 nucleotides.

3. A primer according to one of the claims 1 and 2, characterized in that it comprises one of the following sequences :

SEQ ID1 (PL3)   AAG TTA CCT TTG CTG CAT AAT CCC
SEQ ID2 (PL7)   ATA GAT ACT CCA TAT CAT CCT GCT
SEQ ID3 (Plc)   TCA AAA GAA TAT GCA AGA GGT
SEQ ID4 (PL1)   TTCTAT CTT GGA GAGG CTATGCAC
SEQ ID5 (PL4)   GCTACTAGTTCTTTTACATTCTTTCC.

4. A primer for the amplification of at least one part of the gene encoding the enterotoxin of C. perfringens, characterized in that it corresponds to a part of the sequence comprised between the nucleotides 450 and 950 and preferentially between the nucleotides 450 and 550, or between the nucleotides 750 and 950 of the said gene, and in that it is able to amplify a part of the said gene, by cooperating with another primer having similar features and a reversed polarity.

5. A primer according to claim 4, characterized in that it comprises almost from 10 to 30 nucleotides.

6. A primer according to one of the claims 4 and 5

36

characterized in that it has one of the following
sequences:
SEQ ID6 (P145)   GAA AGA TCT GTA TCT ACA ACT GCT GGT CC
SEQ ID7 (P146)   GCT GGC TAA GAT TCT ATA TTT TTG TCC AGT

5   SEQ ID8 (Ent A)   GAA CGC CAA TCA TAT AAA TTT CCA GCT GGG

        7. A process for the detection of the gene encoding
the lecithinase in a sample, comprising the following
steps:
        - DNA from the sample is isolated ,

10   - the parts of the genes encoding the lecithinase
are amplified by chain polymerisation with the help of
specific primers, such as defined in one of the claims 1
to 3,
        - the amplification products are determined with

15   the help of known methods,
        8. A process for the detection of the gene encoding
the enterotoxin in a sample, comprising the following
steps:
        - DNA is isolated from the sample,

20   - the parts of the genes encoding the lecithinase
are amplified by chain polymerisation with the help of
specific primers, such as defined in one of the claims 4
to 6,
        - the amplification products are determined with

25   the help of known methods,
        9. A process for the determination of the presence
and the numeration of C. perfringens in a sample ,
comprising the following steps:
        - DNA from the sample is isolated ,

30   - parts of the genes of C. perfringens encoding
respectively the lecithinase and the enterotoxin are
simultaneously amplified by chain polymerization withe the

37

help of specific primers , such as defined in one of
claims 1 to 6 ,
        - the amplification products are determined with
the help of known methods.

5   10. A process according to one the claims 6 to 9
wherein the DNA is isolated from a food sample according
to the following steps:
        - the sample is incubated in a medium allowing the
growth of C. perfringens,
10   - the bacteriae are separated from the food
particles par centrifugation,
        - the bacteriae are put in contact with a resin
lysing them, and
        - the DNA is separated from the bacterial
15 fragments.
        11. The C. perfringens beta toxin gene, called β_2
gene, isolated from the C. perfringens strains of C type.
        12. The plasmids corresponding to the gene of claim
11 and registered in the CNCM Collection under the
20 respective references I-1499 and I-1500.

38
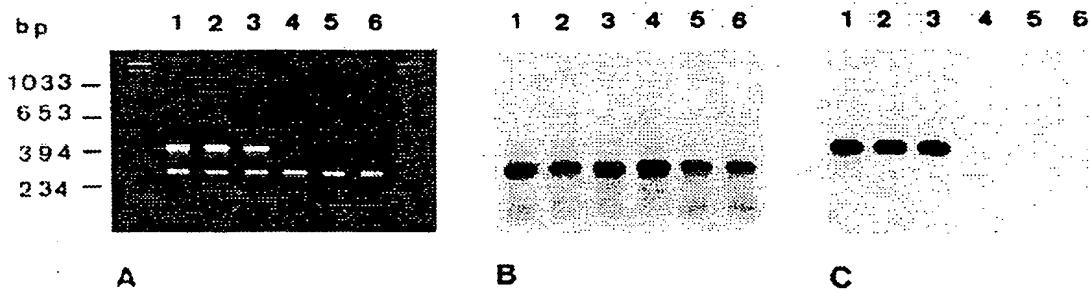
FIGURE 1

peptide 21   (N)- Y - D - I - N - T - V - V - N - I - S - E - D - E
P221   5'-TAT GAT ATI AAT ACI GTI GTI AAT ATI TCI GAA GAT GAA-3'

peptide 23   V - M - E - N - Y - L - N - A - L - K Y - I - L - T - P -S - F
             GTI ATG GAA AAT TAT TTI AAT GCI TTI AAA
P263   3'-CAI TAC CIT TTA ATA AAI TTA CGI AAI TT-5'

N-terminal   K - E - I - D - A - Y - R - K - V - M - E - N - Y - L - N - N - A - L
P268   5'-AAA GAA ATI GAT GCI TAT AGI AAA GTI ATG GAA AAT TAT TTI AAT GC-3'
P279   5'-AAA GAA ATI GAT GCI TAT AGI AAA GTI ATG G-3'

peptide 13   N - F - T - P - A - S - I
             AAT TTI ACI CCI GCI TCI AT
P280   3'-TTA AAA TGI GGI CGI AGI TA-5'

FIGURE 2: Internal and N-terminal protein microsequencing of the purified beta toxin
           from strain CWC 245 and corresponding synthetic oligonucleotides .

FIGURE 3: Partial nucleotide sequence and translation
into amino acids of beta 2 .

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1    AAA GAA ATG GAT GCG TAT AGG AAA GTG ATG GAG AAT TAT CTT AAT GCT    48
1    K   E   M   D   A   Y   R   K   V   M   E   N   Y   L   N   A      16

49   TTA AAA AAC TAC GAT ATT AAT ACA GTT GTA AAC ATT TCA GAA GAT GAA    96
17   L   K   N   Y   D   I   N   T   V   V   N   I   S   E   D   E      32

97   AGA GTA AAT AAT GTT GAA CAG TAT AGA GAA ATG TTA GAA GAT TTT AAA   144
33   R   V   N   N   V   E   Q   Y   R   E   M   L   E   D   F   K      48

145  TAT GAT CCT AAC CAA CAA CTG AAA TCT TTT GAA ATA CTT AAT TCA CAA   192
49   Y   D   P   N   Q   Q   L   K   S   F   E   I   L   N   S   Q      64

193  AAG AGC GAT AAT AAA GAA ATA TTT AAT GTA AAA ACT GAA TTT TTA AAT   240
65   K   S   D   N   K   E   I   F   N   V   K   T   E   F   L   N      80

241  GGT GCA ATT TAT GAT ATG GAA TTT ACT GTA TCA TCT AAA GAT GGA AAA   288
81   G   A   I   Y   D   M   E   F   T   V   S   S   K   D   G   K      96

289  TTA ATA GTA TCT GAT ATG GAA AGA ACA AAA GTT GAG AAT GAA GGA AAA   336
97   L   I   V   S   D   M   E   R   T   K   V   E   N   E   G   K     112

337  TAT ATT TTA ACA CCA TCA TTT AGA ACT CAA GTT TGT ACA TGG GAT GAT   384
113  Y   I   L   T   P   S   F   R   T   Q   V   C   T   W   D   D     128

385  GAA CTA GCA CAA GCA ATT GGG GGA GTT TAT CCA CAA ACA TAT TCT GAT   432
129  E   L   A   Q   A   I   G   G   V   Y   P   Q   T   Y   S   D     144

433  AGA TTT ACA TAT TAT GCA GAT AAT ATA TTA TTA AAC TTC AGA CAA TAT   480
145  R   F   T   Y   Y   A   D   N   I   L   L   N   F   R   Q   Y     160

481  GCA ACT TCA GGT TCA AGA GAT TTA AAA GTA GAA TAT AGT GTT GTA GAT   528
161  A   T   S   G   S   R   D   L   K   V   E   Y   S   V   V   D     176

529  CAT TGG ATG TGG AAA GAT GAT GTT AAA GCT TCT CAA ATG GTA TAT GGT   576
177  H   W   M   W   K   D   D   V   K   A   S   Q   M   V   Y   G     192

577  CAA AAT CCT GAT TCT GCT AGA CAA ATA AGA TTA TAT ATA GAA AAA GGA   624
193  Q   N   P   D   S   A   R   Q   I   R   L   Y   I   E   K   G     208

625  CAA TCT TTC TAT AAA TAT AGA ATA AGA ATT AAA AAC TTT ACA CCT GCA   672
209  Q   S   F   Y   K   Y   R   I   R   I   K   N   F   T   P   A     224

673  TCA ATT AGA GTA TTT GGT GAA GGG TAT TGT GCA TAG AAA AAA ATA TGA   720
225  S   I   R   V   F   G   E   G   Y   C   A   *                    236

721  AGT GAC TTA GTC ACT TCA TAT TTT TTT TAC TAT TAA TTT TAT TAT ATA   768

769  AAA ACC TAA CAT ACA TGA AAG TAT TCT TAA TAC AGT TAT ATC AAA ATT   816

817  AAA GTA GGG GAA ATA AAA TAA AAG GCT AAA AAC TAT ATT AAA AAC TAT   864

865  AAA AAT TAT TAA ATT AGC                                           882
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3/4

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FIGURE 4: Comparison of amino acid sequences of the beta 2 (upper line) and beta 1 (lower line) toxins using the GCG Gap program .

Gap Weight: 3.000   Average Match: 0.540
Length Weight: 0.100   Average Mismatch: -0.396

Quality: 73.4   Length: 338
Ratio: 0.326   Gaps: 5
Percent Similarity: 38.117   Percent Identity: 15.695


```
1     ...............................KEMDAYRKVMENYLNALKN  19
                                     |.. |.  .:..:..
1     MKKKFISLVIVSSLLNGCLLSPTLVYANDIGKTTTITRNKTSDGYTIITQ  50

20    YDINTVVNISEDERVNNVEQY......REMLEDFKYDPNQQLKSFEILNS  63
        |...:  | |.. .| :.:      | : :.:. :  :.  :::.|
51    NDKQIISYQSVDSSSKNEDGFTASIDARFIDDKYSSEMTTLINLTGFMSS  100

64    QKSDNKEIFNV...............KTEFLNGAIYD........ ....  85
        .|.|   . :|:              ...:||:.| :
101   KKEDVIKKYNLHDVTNSTAINFPVRYSISILNESINENVKIVDSIPKNTI  150

86    ....MEFTVSSKDGKLIVSDMERTKVENEGKYILTPSF...........  119
          :.. |:: | |  | | :  ::.|.. |:.|  ....:
151   SQKTVSNTMGYKIGGSIEIEENKPKASIESEYAESSTIEYVQPDFSTIQT  200

120   ..RTQVCTWDDELAQAIGGVYPQTYSDRFTYYADNILLNFRQYATSGSRD  167
        .|  ..||..:.:.. | |   . :.. |::::::  |  ....: :
201   DHSTSKASWDTKFTETTRGNYNLKSNNPV..YGNEMFMYGRYTNVPATEN  248

168   LKVEYSVVDHWMWKDDVKASQMVYGQNPDSARQIRLYIEKGQSFYKYRIR  217
        : .:| : .       :.. | :: :.|..... |:: :|::.. |  ..
249   IIPDYQMSKLITGGLNPNMSVVLTAPNGTEESIIKVKMERERNCYYLNWN  298

218   IKNFTPAS.......................................  225
        |:...
299   GANWVGQVYSRLAFDTPNVDSHIFTFKINWLTHKVTAI  336
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4 / 4